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ZENECA Limited
15 Stanhope Gate
LONDON W1Y 6LN, Great Britain
Patents ADP number *(if you know it)*
6254007002
If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention
DEVICE

5. Name of your agent *(if you have one)*
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"Address for service" in the United Kingdom to which all correspondence should be sent
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Intellectual Property Department
ZENECA Pharmaceuticals
Mereside, Alderley Park, Macclesfield,
Cheshire, SK10 4TG, Great Britain
Patents ADP number *(if you know it)* 1030618002

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Number of earlier application	Date of filing *(day / month / year)*

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11. I/We request the grant of a patent on the basis of this application.

Signature Lynda M Slack Date 7 Oct 1998

12. Name and daytime telephone number of person to contact in the United Kingdom

Mrs Lynda Slack
01625 516173

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DEVICE

The invention relates to the injection of cells. Specifically to automated devices for injection of large numbers of cells. The invention also includes use of such a device in fields where low throughput of cell injection from current techniques has meant that such uses have not been viable.

Injection of cells is a valuable technique in a limited number of fields, for example *in vitro* fertilisation, and currently is normally carried out manually and individually on each cell. It requires a high level of skill and an experienced operator can only inject in the order of one cell per minute. There are many other fields that would benefit from cell injection of macromolecules, genes, chromosomes, organelles, or any other substance desired to be injected into a cell were it possible to achieve this on a large numbers of cells. Gene therapy, biotechnology, life sciences research, diagnostics, pharmaceutical and agrochemical research are among many fields that would benefit from a high throughput cell injection method.

Currently using manual techniques the cells are suspended in solution and each cell is individually injected by fixing a cell into position by the operator "sucking" the cell onto the end of a narrow pipette. Whilst watching the operation through a microscope the operator then inserts a needle into the cell. Once the injection is made the needle is retracted manually and the cell released, then the next cell is fixed and so on. In addition variations of this basic manual technique are available such as for injecting cells which are attached to a dish as a monolayer. The cost of injecting a small number of cells is expensive and means that microinjection of cells is not a technique used widely in the pharmaceutical or agrochemical industry.

We have found that large numbers of cells (thousands or millions) may be micro-injected with minimal operator involvement by use of a microfabricated device which impels cells onto an injection needle.

Microfabrication techniques are generally known in the art using tools developed by the semiconductor industry to miniaturise electronics, it is possible to fabricate intricate fluid

systems with channel sizes as small as a micron. These devices can be mass-produced inexpensively and are expected to soon be in widespread use, for example, in simple analytical tests. See, e.g., Ramsey, J.M. et al. (1995), "Microfabricated chemical measurement Systems," Nature Medicine 1:1093-1096; and Harrison, D.J. et al (1993), "Micromachining a miniaturized capillary electrophoresis-based chemical analysis system on a chip," Science 261:895-897.

Miniaturisation of laboratory techniques is not a simple matter of reducing their size. At small scales different effects become important, rendering some processes inefficient and others useless. It is difficult to replicate smaller versions of some devices because of material or process limitations. For these reasons it is necessary to develop new methods for performing common laboratory tasks on the microscale.

Devices made by micromachining planar substrates have been made and used for chemical separation, analysis, and sensing. See, e.g., Manz, A. et al. (1994), "Electroosmotic pumping and electrophoretic separations for miniaturized chemical analysis system," J. Micromech. Microeng. 4:257-265.

We have found that by careful arrangement of channels formed within a microfabricated device (microfluidic channels) a conduit is made within which the flow of cells in a suspension may be controlled to an extent that cells may be individually injected by impelling them onto an injection needle.

We disclose as the first feature of the invention a microfabricated cell injector comprising an internal surface defining a conduit for transporting cells suspended in a fluid, and having an inlet and an outlet, the conduit further comprising a cell injection needle for injecting material into cells as an internal projection, such that in use cells enter the injector via the inlet and are moved along the conduit, pierced by the cell injection needle whereupon material is injected into the cell, and then moved to the outlet.

A further feature of the invention is a method for the microinjection of cells which method comprises passing a suspension of cells in a fluid through a conduit comprising a cell injection

needle as an internal projection, the cells thereby being pierced by the injection needle and material injected into the cell as the cells pass through the conduit.

It should be understood that the arrangement, type and dimensions of the device and the components therein will vary according to the use or application, as will become apparent. It is preferred that the microfabricated conduit only allows a single cell to pass a single injection needle at any one time.

In this disclosure, the term "microfabricated" includes devices capable of being fabricated on silicon wafers readily available to those practicing the art of silicon microfabrication and having the feature sizes and geometries producible by such methods as LIGA, thermoplastic micropattern transfer, resin based microcasting, micromolding in capillaries (MIMIC), wet isotropic and anisotropic etching, laser assisted chemical etching (LACE), and reactive ion etching (RIE), or other techniques known within the art of microfabrication. In the case of silicon microfabrication, larger wafers will accommodate a plurality of the devices of this invention in a plurality of configurations. A few standard wafer sizes are 3" (7.5cm), 4"(10cm), 6"(15cm), and 8"(20cm). Application of the principles presented herein using new and emerging microfabrication methods is within the scope and intent of the invention.

The injection needle has a diameter of dimensions comparable with the dimension of the cells to be injected, for example between 1% and 50% of the cell diameter, preferably between 5% and 30%. The walls of the injection needle are around 1 micron thick and may be as narrow as 0.1 micron thick. The injection needle is connected to a microfluidics channel which is able to deliver to the injection needle the sample for injection. Preferably the injection needle is fixed in the device relative to the walls of the micofluidic channels such that it projects into the conduit and injection is achieved by moving the cells on and off the injection needle, rather than by moving the injection needle into and out of the cell. Preferably the injection needle is positioned on a surface of the microfluidic channel which we term the "injection wall" , see for example Figure 1. The length of the injection needle that is exposed above the injection wall will determine the "injection depth", that is the depth to which the injection needle will penetrate the cell. This depth will depend on the cell type and the application. In particular, it will depend on the cellular compartment that it is desired to inject into. For example, for

injection into the cytoplasm, the injection depth will be in the order of, or less than, 1 micron; whereas, for an injection into the nucleus, the injection depth will need to be greater, for example 3-10 microns. Given a knowledge of the cell type, it will be possible for the skilled bioscientist to select a device with the appropriate injection depth. The injection needle may be a fabricated separately from the microfabricated conduit and inserted during manufacture into the device. Alternatively the injection needle is fabricated during the manufacture of the conduit and the injection needle formed as a simple projection from the surface of the injection wall, see for example Figure 8.

The injection wall surrounding the immediate area of the injection needle may be permeable to the medium in which the cells are contained, but impermeable to the passing of cells. In certain orientations of the device permeable walls are preferred and allow the passing of the cell medium through the injection wall, facilitating the movement of the cell onto the injection needle. The injection wall may itself be charged to attract the cell onto the injection needle, or reverse charged to then expel the cell from the injection needle. The injection wall may be flat or any other shape to accommodate the cell whilst it is on the injection needle.

Cells may be suspended in any liquid which is able to readily flow through microfluidic channels and not adversely harm the cells suspended within the liquid. Suitable liquids are buffered aqueous solutions optionally containing cell nutrients.

By high throughput we mean that the invention can achieve a throughput substantially higher than conventional means and that numbers of cells in excess of 100, for example 1,000 or 1 million, can be achieved in a convenient time period in the order of minutes or hours. In order to achieve the higher throughputs, the method optionally involves parallel processes, i.e. multiple devices are used in parallel and cells are flowed along a plurality of microfluidic channels such that they are impelled onto a plurality of injection needles, for example, one injection needle per channel. Figure 6 shows such a device with 8 channels in parallel. A requirement of such devices, which we term "multi-channel" is that the inlets of the multiple injector units are connected to suitable channels to divide up the flow of cells from a cell sample reservoir, then, preferably, recombined after injection, and the material for injection is also divided up by suitable channels to provide material to each injection area.

Accordingly we disclose as the second feature of the invention a microfabricated device containing a plurality of cell injector units, each cell injector unit comprising a conduit or an internal surface defining a conduit for transporting cells suspended in a fluid, and having an inlet and an outlet, the conduit further comprising a cell injection needle as an internal projection, such that in use cells enter the injector via the inlet and are moved along the conduit, pierced by the injection needle whereupon material is injected into the cell, and then moved to the outlet, the respective inlets and outlets of the cell injecting units being each connected such that the cells are divided into each injector unit and, preferably, recombined after injection.

The injection material is any material that it is desired to inject into the cell. Most advantageously, this is material that cannot readily be taken up by the cell of interest by any other convenient means. In particular, the material for injection is a macromolecule in aqueous solution, for example a peptide, protein, nucleic acid or polysaccaharide, and analogues and conjugates thereof. Also the injection material may comprise particles, for example viruses, chromosomes, synthetic particles optionally containing or coated with a macromolecule of interest, spores, plasmids, cell organelles, vesicles, liposomes, micelles and emulsions. Optionally a label, for example a fluorescent label, may be added to the injection fluid to act as a marker to indicate that the injection is successful.

Cells may be moved along the microfluidic channels and impelled onto the injection needle by any convenient means. Two broad categories are envisaged. Firstly is passive flow, where the carrier fluid containing the cells of interest is moved and the cells flow with it. The carrier fluid may be propelled by means of a mechanical pump, by applying a vacuum or pressure to one end of the channel, by gravity flow or by electro-osmosis. A preferred method is electro-osmosis, which may conveniently be achieved by the microfabrication of electrodes at the ends of the channels, the voltages between electrodes being controlled conveniently externally to achieve the desired fluid movements. Alternatively active flow may be employed where the cell is moved actively, i.e. independently of the carrier fluid by means of an external field, for example an electrostatic field. Means of manipulating cells by various types of electric field are described in the literature. Combinations of methods are also possible, for example, the cells

may be delivered to the injection area of the device by passive flow and then impelled by an active force onto the injection needle.

The above cell movement methods are also used, optionally in combination, to remove the cell from the injection needle and to flow the cells away and out of the device.

Where the cells are manipulated onto or off the injection needle by passive flow, the injection wall may be alternatively constructed with holes or small channels therein, or is of a porous material, so that fluid can flow relatively unhindered through the wall. Figure 2 illustrates this arrangement.

The arrangement of microfluidic channels to effect the method of the invention will depend on many factors such as the desired throughput and means of propelling cells.

In the case of passive flow and use of a porous injection wall, a convenient configuration is shown in Figure 3. Here a stream of cells enters along channel A (at this point in the cycle there is no flow between arms B and C) and the leading cell becomes impaled on the injection needle. A "capture sensor" senses that a cell is captured and the flow is halted and the material for injection is injected. The flow is immediately reversed in a short pulse which dislodges the cell. The strength and duration of this pulse is selected such that the cell is delivered to the centre of the cross. Flow from arm B to arm C (the reverse is possible) is initiated and the cell is removed along arm C. The cycle then starts again with flow down channel A which capture the next cell, the said cycles being repeated until the desired number of cell have been injected.

A suitable capture sensors include a conductivity sensor that measures the conductivity between the injection fluid and the carrier fluid. This will change when the needle penetrates the cell. Optionally, the change in impedance may be measured to detect a cell that is adhered to the needle but not actually penetrated by it. Alternatively, the capture sensor may take the form of a pressure transducer positioned near the point of capture such that capture causes a partial blockage of the flow in the pressure transducer and a change in pressure. Alternatively,

the capture sensor may take the form of a pair of electrodes positioned either side of the cell when in the injection position, the electrodes being able to measure changes in conductivity or another convenient electrical parameter. Alternatively, optical methods may be used to image the cell or detect its presence at the injection position by changes in absorbance, refractive index, light scattering and the like.

Alternatively, cells may be introduced along arm B as is illustrated in Figure 4.

It will be appreciated that, depending on the cell density and the efficiency of injection required, it may be desirable to have a means of detecting the presence of a cell as it approaches or enters the injection area, i.e. a "cell sensor". This may be achieved by any convenient means for example by a pair of electrodes positioned either side of the cell when in the position for detection, the electrodes being able to measure changes in conductivity or another convenient electrical parameter. Alternatively, optical methods may be used to image the cell or detect its presence at the desired position by changes in absorbance, refractive index, light scattering and the like.

A further way of impelling the cells onto the injection needle is by deflecting the flow at a substantial angle, for example between 40 degrees and 90 degrees such that the cell impacts a defined area of the channel wall which contains the injection needle. Removal of the cell is achieved by a combination of the elasticity of the cell bouncing off the wall and the flow in the microfluidic channel, optionally enhanced by a tumbling action of the cell initiated by geometric features fabricated in the channel wall. A non-limiting illustration of this arrangement is shown in Figure 5.

The walls of the channel are designed such that each cell is presented to the injection needle with the correct force such that the injection needle penetrates the cell wall, and that the cell then bounces off the wall and continues in the same direction down the channel, without tearing or otherwise irreparably damaging the cell wall. To achieve this, the channel walls have several features to achieve this. We here define the various surfaces and features (see Figure 5)

a "deflector wall", which causes the liquid flow and the cells to deviate

a "constrictor" which squeezes the cell slightly and increases the speed of the cell. The constrictor also encourages the cell to proceed down the channel with a tumbling action.

When the cell is positioned on the needle, the injection of the injection fluid takes place. The amount of fluid injected will depend on the cell type, volume and purpose of the injection. It will normally be in the range of 1% to 50% of the cell volume, for example 5% to 20%, and will typically be in the order of one or a few picolitres. Movement of fluid in the injection channel may be achieved by any convenient means and may include for example a micropump or piezoelectric displacement, such as described in Transaction on Biomedical Engineering (1975) 22, 5, 424-426. The movement may optionally be continuous where it is acceptable for injection fluid to leak into the carrier fluid and where this is not economically prohibitive.

Devices may be made by the use of microfabricated layers such as shown in Figure 8. Here the cells travel in suspension down a channel defined by the gap between layers 3 and 4, and by walls perpendicular to the layers, formed from layer 3 or 4 or both. As each cell enters the injection area, flow away from the injection area is initiated in the channel formed between layers 2 and 3. This impels the cell onto the injection needle. This flow may optionally be maintained while injection takes place, whereafter the flow is reversed thereby ejecting the cell from the injection needle into the main cell flow. The main cell flow may optionally be paused while the cell is drawn onto the injection needle, is injected and released. Alternatively, the sequence of flows may be as follows: 1) channel A to channel C until a cell is detected at the injection needle 2) injection 3) channel C to channel B until cell is ejected 4) repeat i.e. go to 1). The length of step 3) will depend on experience of what is required to eject the cell and the desired concentration of the cells coming from the device, it being understood that the device could concentrate or dilute the cells depending on the relative timing of the flow steps.

The relative size and shape of the features and other aspects of the injector device will depend on numerous factors. This will include the type and size of cells being injected, the desired efficiency of injection (i.e. the percentage of cells that are successfully injected), the desired throughput, flow rate, clumping tendency of the cells and other factors.

The device may be fabricated in glass, silicon, plastic or any other suitable material or combination of materials using conventional microfabrication techniques. It will be appreciated that for each material the constraints imposed by the material and manufacturing technique used may require different geometries to those shown in the diagrams contained herein.

We have further found that microfabrication technology offers an approach to optimise the various parameters to suit the cells type and the application. At the design stage, one or more devices are fabricated that have injection areas of different geometries and different arrangements of design elements in a large number of combinations. It is then a straightforward matter to inject cell populations down the different injection areas and determine empirically which arrangement gives satisfactory results according to whatever success criteria are considered important.

It will be clear to the microtechnologist that there are many possible arrangements of the conceptual and material elements of the present invention, and analogues or equivalents thereof, that may be expected to yield a device and method for achieving high throughput injection of cells.

The use of the method and device of the invention are numerous in the fields of, for example, life science research, medical research, drug and agrochemical discovery, diagnostics.

The principle advantage is that the device provides a means of reliably injecting a wide variety of material. Thus it may be used as a generic and predictable transfection method which allows genetic material to be injected into cells with high efficiency. This is of particular advantage

when there is a need to transfect cells that are difficult to transfect by conventional means. It is also of advantage when it is required to transfect cells with two or more genes.

The method of the invention may also be used for validating assays used for testing modulation of a biological target. In particular, the situation often arises that the only material that can validate an assay, that is provide a control, is material that cannot be readily provided to the cell interior. The method provides a way of reliably injecting a wide variety of samples.

The device and method of the invention may also be used for validating whether a biological target does or does not influence an important cellular process. In particular the device and method allow for inhibitory antibodies and dominant negative proteins to be incorporated inside the cell in order to ascertain the relevance of target proteins, interactions, enzyme activity and pathways to potential therapeutic intervention.

The device and method of the invention may also be used for the construction of intracellular assays. In particular, protein and other non-permeable agents or probes, in particular labelled agents or probes may be incorporated into a population of cells that may subsequently be employed for testing and evaluating compounds. In particular labelled antibodies may be injected. Probes may be based on numerous assays principles, for example fluorescence radiation energy transfer (FRET) and fluorescence polarisation, or may be specifically designed to modulate their signal in the presence of a target molecule or enzyme activity.

The device and method of the invention may be used to evaluate compounds of pharmaceutical or agrochemical interest, especially in cases where there are concerns about the ability of the compound to penetrate the cell. The device allows all such compounds to be reliably incorporated into the cell and reach the site of action.

The device and method of the invention may be used to ascertain the sensitivity of cells to certain compounds which are injected. This will be of value in determining which compound should be employed to treat a particular condition.

The method of the invention may also be used for *ex vivo* therapy, for example *ex vivo* gene therapy. Here a population of cells from a human subject may be removed, microinjected using the device and method of the invention and replaced into the subject.

It will be clear that for certain applications, having achieved injection on a microfabricated device, it may be advantageous to incorporate subsequent process or analysis steps on the same device.

Accordingly, we disclose an integrated cell process device which comprises the cell injection function combined with one or more process or analysis steps.

Process steps may include for example further injections, cell fusion, FACS (fluorescence-activated cell sorting) or other cell sorting, or incubation with a further agent and use in a biological assay. The biological assay may include for example an assay for the biological activity of one or more test compounds.

Analysis may include flow cytometry.

The process or analysis steps may either precede or follow the injection step, or both. In the case of FACS, this may sort the cells into different channels where they are injected with a different injection material. Subpopulations may optionally be pre-marked for FACS sorting.

A further application of the invention is that it may be used as a means of extracting material from inside cells. This may be achieved by simple reversing the flow in the injection channel. This ability may be exploited either to harvest an intracellular cell product, which may be for example a protein or genetic material, or it may be used to sample cell contents for subsequent analysis. Further uses of this facility for extracting cells contents will be apparent to the scientist of average skill in various fields.

The device is also able to be used to measure the electrical properties of cells for example by means of electrodes placed in the injection channel and the channel containing the cells. For

example, the membrane potential and membrane permeability may be measured on populations of cells, optionally in response to external agents or test compounds.

Manufacture

Microfabricated devices of the invention may be prepared by standard techniques currently employed by microtechnologists. By way of example the following suggested route for manufacture of the device in Figure 8 is provided.

The injection needle has a height in the order of 3 micron above the upper surface of layer 2, the walls are approximately 1 micron thick and the internal diameter of the needle is 1 micron at its narrowest point.

Several methods for fabrication of the needle could be devised by a skilled technologist. For example, layer 2 could be fabricated from glass or silicon by 1) patterning to give cylindrical resist cores in the desired positions on the chip, 2) sputtering with glass or metal over the cores at an angle away from normal to cover the sidewalls, 3) planarising with resist and ion milling off the top to leave a core surrounded by an open cylinder of glass or metal, 4) removing the resist to leave a cylinder and 5) anisotropically etching from the rear to connect with the hole in the centre of the cylinder. It will be understood that other features such as channel walls, spacers, electrodes etc. will also be incorporated during the fabrication of the needle feature.

Various techniques may be employed to sharpen the tip of the injection needle to aid penetration. For example milling techniques may be employed. Alternatively, the 5-step method described above may be elaborated by the provision of a thin metal disc, of diameter slightly greater than the resist cores, positioned centrally on top of the resist cores. The sputter coating to form the sides of the cylinder is then carried out from above at an angle such that the overlap casts a "shadow" on the sides of the resist, giving a sloping internal surface with the wall tapering to the top. The metal disc and resist are then removed and the etch from the rear step carried out as before.

The invention is described in more detail below in the following non-limiting figures.

Figure 1 - The depth of entry of the injection needle **2** into the cell **1** is defined by the distance the cell may travel before it is stopped at the cell injection wall **5**. Material **3** may be injected into the cell by pulsed injection when the cell is in position, or by continuous flow where the time the cell is spent on the injection needle is regulated.

Figure 2 - Shows one alternative method for impelling the cell onto the injection needle and removal once injection has taken place. The cell is impelled onto the injection needle **1** and **2** by a passive force, i.e. the liquid and cell moves, or an active force, i.e. where the cell moves. Movement of the cell onto the injection needle is eased by providing a permeable cell injection wall for the cell suspending liquid to pass through. The cell is removed from the injection needle by providing an opposite active or passive force **3**.

Figure 3 - Is a diagram of a microfabricated cell injector with a conduit consisting of two channels crossing in opposing directions. Cells are impelled down channel A to D stepwise by an oscillating force which switches cell movement from A to D then D to A, the movement of cells from A to D being larger then the return movement and impelling a cell onto the injection needle 1. The smaller returning force D to A releases the cell from the injection needle after injection and places it in line with channel B to C. The cell is moved along B to C and then a force applied again to impel the next cell onto the injection needle in direction A to D.

Figure 4 - Shows an alternative arrangement of the microfabricated cell injector of figure 3 where the cells are moved along channel B to C in a stop/start motion where a cell placed in-line with channel A to D during the stop phase is impelled onto the injection needle by a force A to D and removed by a force D to A and placed back in line with the channel B to C and removed by the next movement in channel B to C.

Figure 5 - Is a diagram of a microfabricated cell injector with a conduit consisting of a single channel **1**. Cells impelled through the channel are forced onto an injection needle **4** by a deflector **2**. The momentum of the cell is sufficient for it to be impelled onto and then off the injection needle.

Figure 6 - Shows how a number of the devices may be placed in parallel to achieve an even higher throughput. Cells are fed into the microfabricated injectors **1** by a series of splitting channels, one for each injector, and then the resulting injected cells collected from all the injectors in a collecting pool **2**. The material for injection is held in a storage area **4** and pumped through to the injection needle tips by a suitable pump **3**.

Figure 7 - Shows an alternative arrangement for the injecting wall **2** which is a narrowing channel which supports the cell **1** during injection.

Figure 8 - Is a diagram of a microfabricated cell injector with a conduit consisting of three parallel channels defined between 4 layers (**Layers 1 to 4**). Cells pass in the direction A to B in the channel defined by **layer 1 and 2**. An oscillating pressure is applied in the second channel **2** which impels a passing cell onto the injection needle **1** and in the opposite direction forces the cell off the injection needle and back into the flow of A to B. Alternatively the flow is from A to C until a cell is detected at the needle and then, after injection, the flow is from C to B for a period. The third and fourth layer define the internal surface of the injection needle and carries the material for injection into the cells. The injection needle has a height in the order of 3 micron above the upper surface of layer 2, the walls are approximately 1 micron thick and the internal diameter of the needle is 1 micron at its narrowest point.

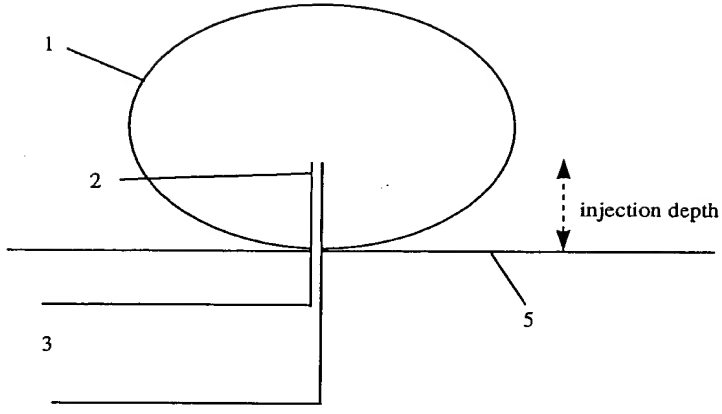


Fig. 1

5

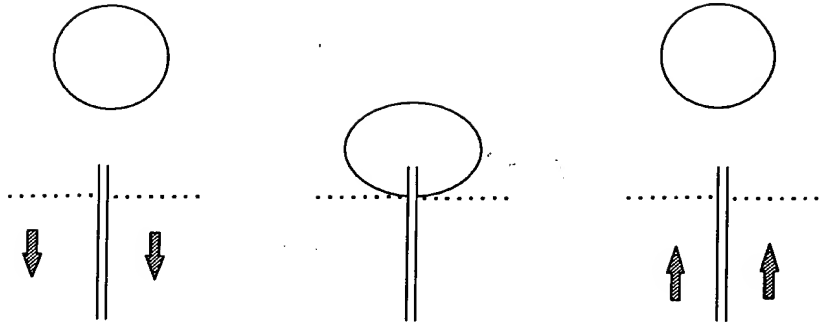


Fig. 2

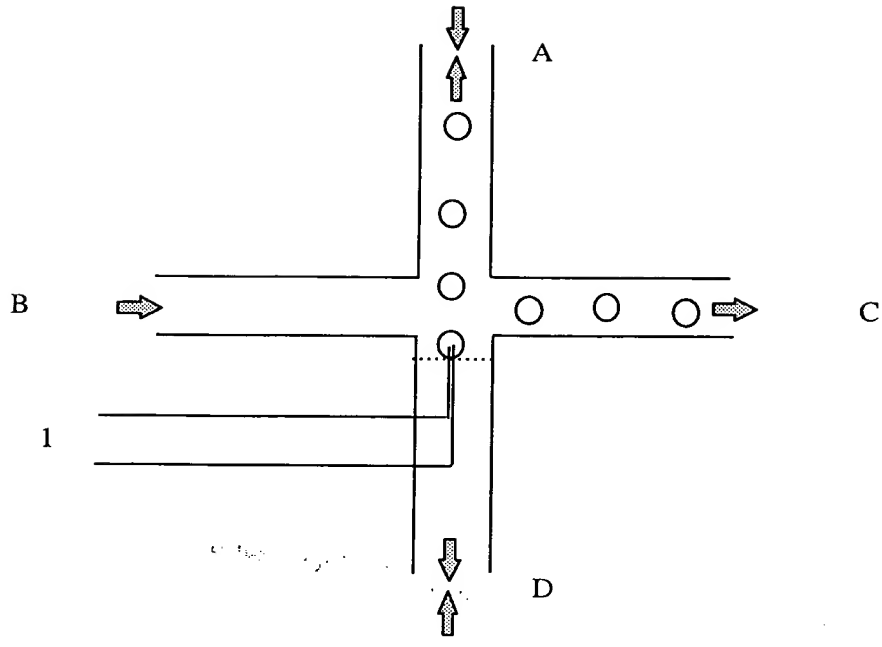


Fig. 3

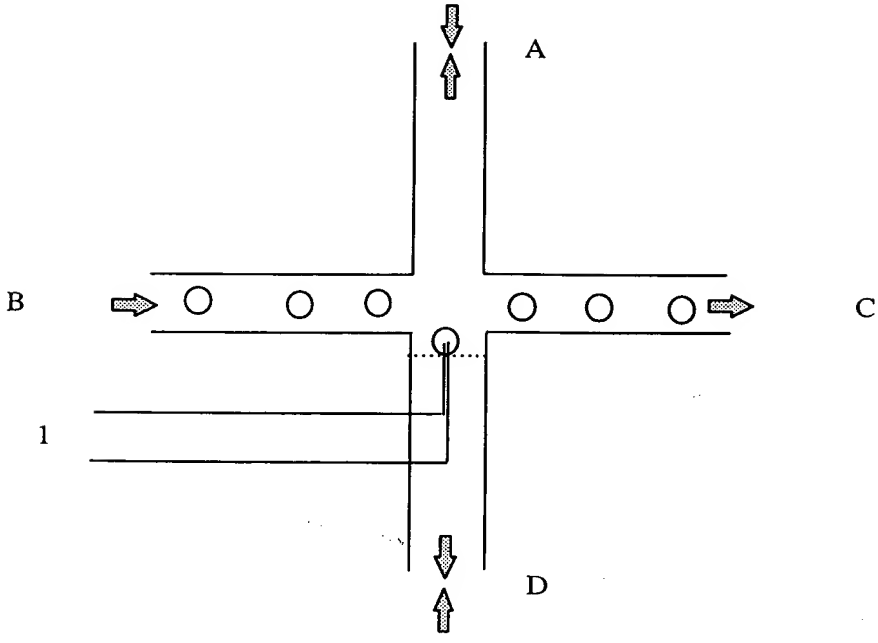


Fig. 4

5

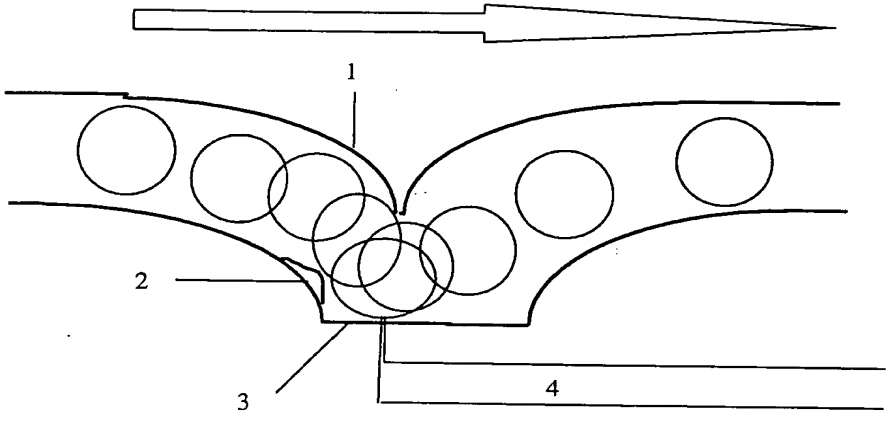
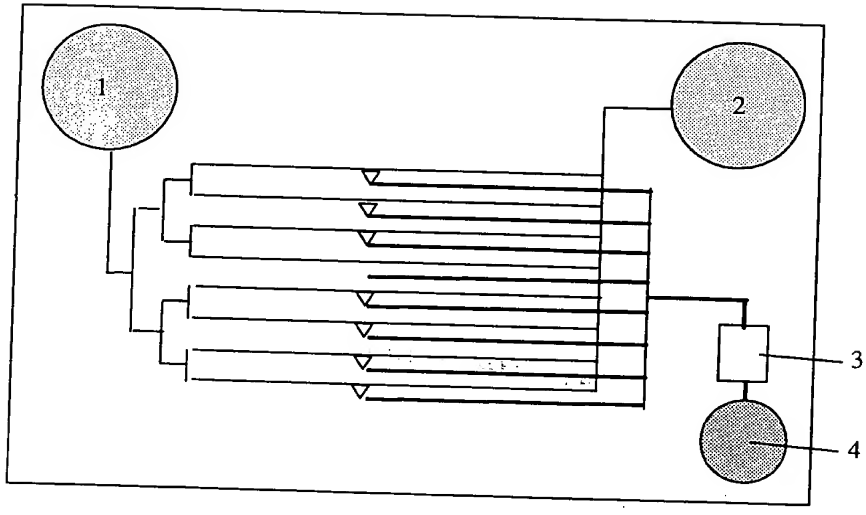


Fig. 5

5



▽ = injection area

Fig. 6

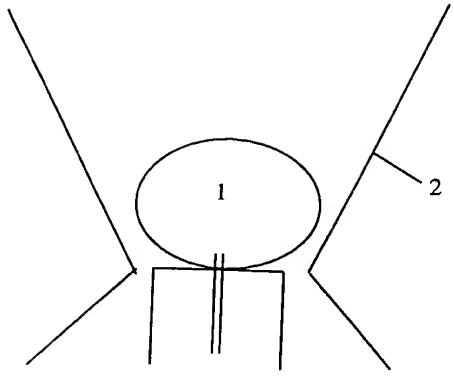


Fig. 7

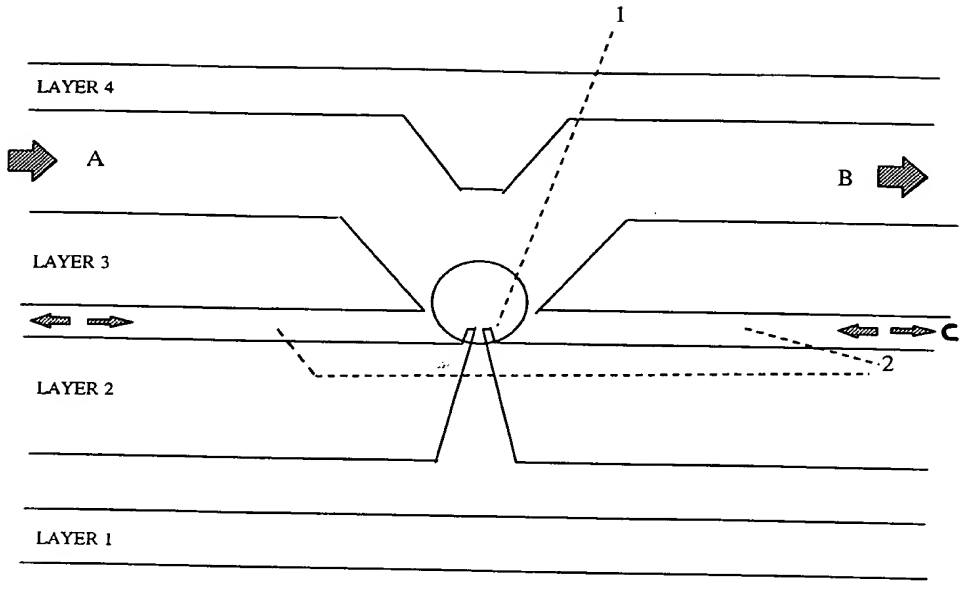


Fig. 8

PCT/GB99/03330

Zeneca

F23 filed 7 Oct 1999